

## Thyroid hormone induces cyclin D1 nuclear translocation and DNA synthesis in adult rat cardiomyocytes

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**ABSTRACT** Although mammalian cardiomyocytes lose their proliferative capacity after birth, there is evidence that postmitotic cardiomyocytes can proliferate provided that cyclin D1 accumulates in the nucleus. Here we show by Northern blot, Western analysis, and immunohistochemistry that 3,5,3'-triiodothyronine (T3) treatment of adult rats caused an increase of cyclin D1 mRNA and protein levels. The increased cyclin D1 protein content was associated with its translocation into the nucleus of cardiomyocytes. These changes were accompanied by the re-entry of cardiomyocytes into the cell cycle, as demonstrated by increased levels of cyclin A, PCNA, and incorporation of bromodeoxyuridine into DNA (labeling index was 30.2% in T3-treated rats vs. 2.2% in controls). Entry into the S phase was associated with an increased mitotic activity as demonstrated by positivity of cardiomyocyte nuclei to antibodies anti-phosphohistone-3, a specific marker of the mitotic phase (mitotic index was 3.01/1000 cardiomyocyte nuclei in hyperthyroid rats vs. 0.04 in controls). No biochemical or histological signs of tissue damage were observed in the heart of T3-treated rats. These results demonstrated that T3 treatment is associated with a re-entry of cardiomyocytes into the cell cycle and so may be important for the development of future therapeutic strategies aimed at inducing proliferation of cardiomyocytes.—Ledda-Columbano, G. M., Molotzu, F., Pibiri, M., Cossu, C., Perra, A., Columbano, A. Thyroid hormone induces cyclin D1 nuclear translocation and DNA synthesis in adult rat cardiomyocytes. *FASEB J.* 20, 87–94 (2006)

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ALTHOUGH IT IS GENERALLY BELIEVED that mammalian cardiomyocytes are unable to divide in the adult, studies have shown that an increase in myocyte number occurs in cases of severe myocardial hypertrophy (1–3). In addition, a small population of cardiomyocytes retaining a proliferative capacity has been shown to be present in regions adjacent to the infarcts in the adult heart (4, 5). Evidence that the adult heart has a limited but significant proliferative capacity raises the possibility that increasing the number of the remaining car-

diomyocytes by activating their proliferative potential could replace damaged myocardium. Improvement in the knowledge of the mechanisms that regulate cell cycle progression may be helpful in finding efficient ways to stimulate cardiomyocyte progression into the cell cycle.

A key role in the control of the cell cycle is played by a complex formation between cyclins and cyclin-dependent kinases (CDKs), which, after activation by phosphorylation, leads to progression into the cell cycle (6, 7). With its CDK partner, each cyclin acts at a different step of the cell cycle, the D-type cyclins, in association with their main partners CDK4 or CDK6, being important in the early G1 phase and cyclin E in association with CDK2, acting in the late phase of G1. The ultimate substrate in this pathway is pRb, the major target of the cyclin D1/CDK4 complex (7, 8). Phosphorylation of pRb by the cyclin D1/CDK4 complex frees the E2F transcription factors, enabling them to *trans-activate* target genes responsible for the progression of the cycle from G1 to the S phase (7, 8). In addition to pRb phosphorylation, cyclin D1 sequesters CDK inhibitors such as p21cip1 and p27kip1 and thereby facilitates activation of the cyclin E/CDK2 and cyclin A/CDK2 complexes required for entry into the cell cycle and its progression to the S phase.

In the heart, evidence that cyclin D1 may play an important role in cardiomyocyte proliferation stems from the following: 1) during cardiomyocyte differentiation, pRb phosphorylation is reduced in association with cell cycle arrest (9); 2) stimuli that induce cardiomyocyte hypertrophy also up-regulate cyclin D1 expression (10, 11), and 3) coinfection of recombinant adenoviruses expressing a variant of cyclin D1 and CDK4 induces pRb phosphorylation and stimulates re-entry into the cell cycle of cardiomyocytes in culture as well as in adult hearts (12). The latter finding suggests that prevention of the nuclear import of the cyclin D1/CDK4 complex plays a critical role as a barrier to prevent cardiomyocyte proliferation; however, it also

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implies that postmitotic cardiomyocytes have the potential to proliferate provided this complex can accumulate in the nucleus.

Thyroid hormone (T3) is a well-known inducer of cardiac hypertrophy (13–15), an adaptive enlargement of the myocardium characterized by the increased size of individual myocytes in response to several extrinsic as well as intrinsic stimuli. However, it is not known whether cardiomyocyte proliferation is involved in this process or whether T3 affects the expression of cell cycle-associated proteins in cardiomyocytes. Induction of cyclin D1 is one of the earlier events associated with hepatocyte proliferation caused by T3, suggesting that this cyclin may represent a critical target in cell proliferation induced by this hormone (16).

In the present study, we addressed the following questions: 1) does T3 induce cyclin D1 expression in the heart? 2) is T3 treatment able to induce nuclear translocation of cyclin D1? and 3) does T3 possess the ability to induce re-entry of cardiomyocytes into the cell cycle?

## MATERIALS AND METHODS

### Animals

Male Fischer F-344 rats (200–250 g) purchased from Charles River (Milano, Italy) were maintained on a laboratory chow diet (Ditta Mucedola, Milano, Italy). The animals were given food and water *ad libitum* with a 12 h light/dark daily cycle and were acclimatized for 1 wk before the start of the experiment. All procedures were performed in accordance with UFAW Handbook on the Care and Management of Laboratory Animals and the guidelines of the Animal Ethics Committee of this university. T3 (Sigma Chemical Co., St. Louis, MO, USA) was added to the diet at a concentration of 4 mg/kg. Rats were killed after 2, 4, and 7 days of treatment. Controls were fed a similar diet in the absence of T3 supplementation. In some experiments aimed at determining the presence of mitotic figures, colchicine (2 mg/kg body weight, dissolved in saline, Sigma Chem. Co.) was injected intraperitoneally 3 h before sacrifice. At the end of the treatment, all the animals were killed and hearts were surgically removed. The degree of hypertrophy was expressed as heart weight (HW) and heart weight/body weight ratio (HW/BW).

### Histology and immunohistochemistry

Immediately after sacrifice, sections of the heart were fixed in 10% formalin and embedded in paraffin. Tissue sections of 5  $\mu$ m thickness were prepared and stained with hematoxylin-eosin. Other sections were used for immunohistochemical detection of cyclin D1,  $\alpha$ -sarcomeric actin, bromodeoxyuridine (BrdU), and phosphorylated histone-3 (H3P).

### Immunostaining of cyclin D1

Briefly, 4 micron-thick sections were deparaffinized and incubated with normal goat serum (Dako Corporation, Carpinteria, CA, USA), mouse anti-cyclin D1 (1:75, clone DSC-6, Dako) and DakoCytomation EnVision<sup>+</sup> horseradish peroxidase (HRP) Mouse ready-to-use (K-4001). The sites of perox-

idase binding were detected with 3,3'-diaminobenzidine (DAB, Sigma Chemical Co.). Sections were counterstained with hematoxylin.

### Double labeling of BrdU and sarcomeric actin

Control and T3-treated rats were given BrdU (1 mg/mL in drinking water) throughout the experimental period (17). Mouse monoclonal anti-BrdU antibody was obtained from Becton Dickinson (Becton Dickinson, San Jose, CA, USA) and the peroxidase method was used to stain BrdU-positive cardiomyocytes. Peroxidase goat anti-mouse immunoglobulin was obtained from DakoCytomation (Dako EnVision<sup>+</sup> HRP Mouse, K-4001). Briefly, 4 micron-thick sections were deparaffinized, treated with HCl 2N for 60 min, then with 0.1% trypsin type II (crude from porcine pancreas, Sigma Chemical Co.) for 20 min, and treated sequentially with normal goat serum (Dako Corporation), mouse anti-BrdU (Becton Dickinson), and DakoCytomation EnVision<sup>+</sup> HRP Mouse ready-to-use. The sites of peroxidase binding were detected with DAB. To localize BrdU-positive nuclei in cardiomyocytes, all sections were costained with an anti-mouse  $\alpha$ -sarcomeric actin antibody (M0874, DakoCytomation, Glostrup, Denmark) and DakoCytomation EnVision<sup>+</sup> System-alkaline phosphatase ready-to-use (K4017). The sites of phosphatase binding were detected with 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT) substrate system (K0598, Dako Corporation). All sections were counterstained with methyl green.

### Northern blot analysis

Twenty to 30  $\mu$ g of heat-denatured total RNA per lane were loaded onto a 1% agarose/formaldehyde gel containing ethidium bromide for RNA detection with a UV lamp and blotted on Hybond-XL-membrane (Amersham, Buckinghamshire, UK). RNA concentration was determined spectrophotometrically at 260 nm. The gels stained with ethidium bromide were photographed to check the quantity and quality of the nucleic acids. In all cases the lanes contained similar amounts of RNA. For cyclin D1, a pcBZ054 plasmid containing a 1.3 kb EcoR1 fragment was used; DNA probes were labeled with [ $\alpha^{32}$ P]dCTP by random priming (Random Priming DNA labeling Kit, Boehringer Mannheim, Mannheim, Germany). The membranes were exposed to an autoradiographic film (Eastman Kodak, Rochester, NY, USA).

### Western blot analysis

Protein extracts for analyses of cyclin A, proliferating cell nuclear antigen (PCNA), and histone H3 were prepared from frozen hearts powdered in liquid nitrogen-cold mortar. Equal amounts of powder from different animals were resuspended in 1 mL Triton lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.4, 135 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 mM NAF, 5 mM iodoacetic acid, 10  $\mu$ g/mL each of aprotinin, pepstatin, leupeptin). Protease inhibitors were added to the isolation buffer to minimize protein degradation during the isolation protocol. Extracts were incubated 30 min on ice, centrifuged at 12,000 rpm at 4°C, and the supernatants were recovered. All inhibitors used were purchased from Boehringer Mannheim GmbH except for PMSF, NaF, and DTT, which were purchased from Sigma Chemical, and Iodoacetic acid from ICN Biomedicals (Irvine, CA, USA). For analysis of cyclin D1 and sarcomeric actin, nuclear and cytosolic extracts were prepared according to Timchenko et al. (18). Protein concentrations of the extracts were determined according to Bradford (19) using bovine

serum albumin as standard (DC Protein Assay, BioRad Laboratories, Hercules, CA, USA). For immunoblot analysis, equal amounts (from 100 to 150 µg/lane) of proteins were electrophoresed on SDS-12% or -8% polyacrylamide gels. Acrylamide and bis-acrylamide were purchased from ICN Biomedicals. After gel electrotransfer onto nitrocellulose membranes (MSI), to ensure equivalent protein loading and transfer in all lanes, the membranes and gels were stained with 0.5% (wt/vol.) Ponceau S red (ICN Biomedicals) in 1% acetic acid and with Coomassie blue (ICN Biomedicals) in 10% acetic acid, respectively. Before staining, gels were fixed in 25% (v/v) isopropanol and 10% (v/v) acetic acid (Sigma Chemical Co.). After blocking in TBS containing 0.05% Tween 20 (Sigma Chemical Co.) and 5% nonfat dry milk, membranes were washed in TBS-T, then incubated with the appropriate primary antibodies diluted in blocking buffer. Whenever possible, the same membrane was used to detect the expression of different proteins. Depending on the origin of the primary antibody, filters were incubated at room temperature with either anti-mouse or anti-rabbit horseradish peroxidase conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were identified with chemiluminescence detection system, as described by the manufacturer (Supersignal Substrate, Pierce, Rockford, IL, USA). When necessary, antibodies were removed from filters by 30 min incubation at 60°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 7.6) and the membranes were reblotted as above.

#### Antibodies

The antibodies used for immunoblotting were mouse monoclonal antibodies directed against cyclin D1 (72-13 G) and PCNA (PC10), from Santa Cruz; mouse monoclonal antibody directed against p27 (Kip1-p27), from Transduction Laboratories (Lexington, KY, USA); mouse monoclonal antibody anti-sarcomeric actin (M0874), from Dako Corporation; rabbit polyclonal antibodies against cyclin A (C-19) and histone H3 (FL-136) purchased from Santa Cruz Biotechnology. Antibody against phosphohistone-3 (H3P on Ser10), was from Upstate Biotechnology (Lake Placid, NY, USA).

#### Assessment of cardiomyocyte DNA synthesis

Sequential transverse sections from the heart of T3-treated and control rats were stained using antibody against BrdU (Sigma Chemical Co.). To localize BrdU-positive nuclei in cardiomyocytes, immunostaining was performed using  $\alpha$ -sarcomeric actin mouse antibody from DakoCytomation. Forty fields per rat were scored at  $\times 1000$  magnification and the number of cardiomyocyte nuclei staining positively for BrdU was counted in each field. The total number of cardiomyocyte nuclei per field was counted, and labeling indexes calculated as number of sarcomeric actin cardiomyocytes with BrdU-positive nuclei/100 cardiomyocyte nuclei. At least 3500 cardiomyocyte nuclei per group were counted. The results are expressed as means  $\pm$  SE of 7 rats/group.

#### Assessment of cardiomyocyte mitoses

Immunostaining with antibody (1:30) to phosphorylated histone-3 (3HP), a mitosis-specific marker (20), was performed in heart sections from T3-treated and control rats, receiving colchicine 3 h before sacrifice. Cardiomyocytes were identified with antibody to  $\alpha$ -sarcomeric actin as above. The secondary antibody for the H3P antibody was a Dako anti-rabbit (Dako Corporation). The number of cardiomyocyte nuclei staining positively for H3P was counted per field at  $\times 1000$

magnification, as well as the total number of cardiomyocyte nuclei per field; mitotic index (MI) was calculated as the ratio H3P sarcomeric actin-positive cardiomyocyte nuclei/1000 cardiomyocyte nuclei. At least 600 fields and 9000 cardiomyocyte nuclei per heart were scored. Results are representative of two different experiments and are expressed as means  $\pm$  SE of 5 rats per group.

#### Serum FT3, CK, LDH, and creatinine analysis

Immediately after sacrifice, blood samples were collected from the inferior vena cava and analyzed for blood chemistries. Briefly, the blood samples were centrifuged at 1500 rpm for 20 min and the serum was tested for free-triiodothyronine (FT3), creatine kinase (CK), lactate dehydrogenase (LDH), and creatinine using a commercially available kit from Boehringer.

#### Statistical analysis

Data are presented as means  $\pm$  SE. Comparison between treated and control groups was performed by Student's *t* test.

## RESULTS AND DISCUSSION

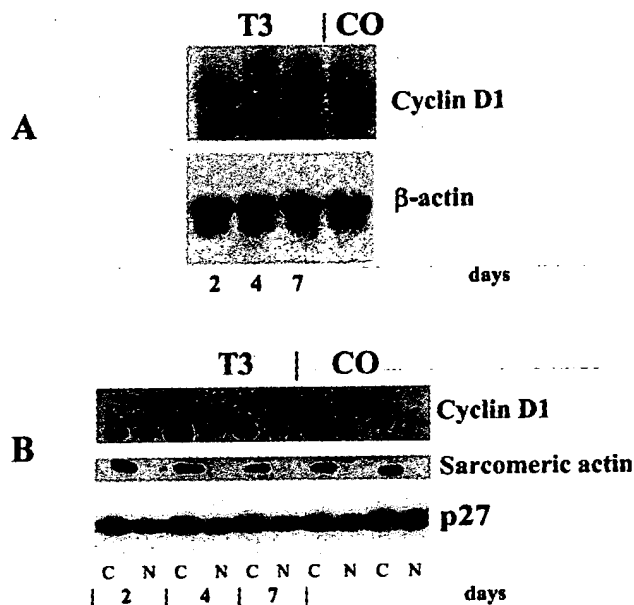
#### Induction of ventricular hypertrophy in T3-treated rats

Determination of serum levels of FT3 in rats treated with T3 showed a very strong elevation compared with controls (44 pg/mL  $\pm$  1.29 in T3-treated rats vs. 3.3 pg/mL  $\pm$  0.24 of controls). Rats treated with T3 for 7 days showed an  $\sim 22\%$  reduction in body weight compared with their untreated counterpart. On the other hand, a strong increase in the size of the heart was seen in treated rats (Table 1). Ventricular cardiac enlargement was evident as soon as 4 days: the combined left/right ventricle weight (HW) and heart weight-to-body weight ratio (HW/BW) after 4 and 7 days of treatment were 44% and 61% larger, respectively, than those in control rats. A similar extent of cardiac hypertrophy caused by T3 was recently shown by De et al. (15).

TABLE 1. Assessment of cardiac hypertrophy in T3-fed rats<sup>a</sup>

|         | Body weight<br>(BW) (g) | Heart weight<br>(HW) (g) | HW/BW%                        |
|---------|-------------------------|--------------------------|-------------------------------|
| Control |                         |                          |                               |
| 2 days  | 202 $\pm$ 2.9           | 0.62 $\pm$ 0.01          | 0.30 $\pm$ 0.015              |
| 4 days  | 208 $\pm$ 1.7           | 0.60 $\pm$ 0.01          | 0.29 $\pm$ 0.003              |
| 7 days  | 223 $\pm$ 8.8           | 0.63 $\pm$ 0.02          | 0.28 $\pm$ 0.002              |
| T3      |                         |                          |                               |
| 2 days  | 195 $\pm$ 3.4           | 0.69 $\pm$ 0.02          | 0.36 $\pm$ 0.007 <sup>s</sup> |
| 4 days  | 188 $\pm$ 1.7*          | 0.80 $\pm$ 0.02*         | 0.42 $\pm$ 0.012*             |
| 7 days  | 180 $\pm$ 3.4*          | 0.80 $\pm$ 0.02*         | 0.45 $\pm$ 0.008*             |

<sup>a</sup>Values are expressed as means  $\pm$  SE of 4 to 6 animals per group. \* Significantly different from controls; *P* = 0.005; <sup>s</sup> Significantly different from controls; *P* = 0.012.



**Figure 1.** A) Induction of cyclin D1 mRNA cardiac levels by T3. Total RNA was prepared from rat heart after 2, 4, and 7 days of treatment with T3. Each lane represents a pool of 4 samples. CO, controls.  $\beta$ -Actin was used as a normalizing control. B) Western blot analysis of cyclin D1, p27, and sarcomeric actin. Protein extracts were prepared as described in Materials and Methods. Appropriate loading was confirmed by staining the gel with Coomassie blue. Each lane represents a pool of 2 samples. C, cytosol; N, nucleus. Similar results were obtained in 3 different experiments.

### Induction of cyclin D1 mRNA by T3

The findings that several stimuli that induce cardiomyocyte hypertrophy up-regulate cyclin D1 expression, and that coinfection of recombinant adenoviruses expressing a variant of cyclin D1 and CDK4 stimulates re-entry of cardiomyocytes into the cell cycle, suggest that cyclin D1 may play a major role in cardiomyocyte-induced DNA synthesis (11, 12). Therefore, cyclin D1 expression was assessed in the heart of rats treated with T3 for 2, 4, and 7 days. As shown in Fig. 1A, the heart of control animals showed only a weak cyclin D1 mRNA expression. On the other hand, cyclin D1 mRNA levels were strongly enhanced in the heart of T3-treated rats with a maximum at 4 days.

### Translocation of cyclin D1 into the nucleus

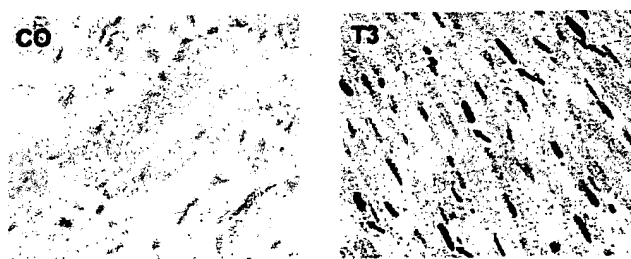
Next, Western blot analysis of the cytosolic and nuclear fractions and immunohistochemistry were performed to determine whether T3 treatment could cause an increase in cyclin D1 protein content and its translocation into the nucleus. As shown in Fig. 1B, in untreated animals cyclin D1 expression was detected in the cytosolic fraction but not in the nuclear fraction. In T3-treated rats, increased levels of cyclin D1 protein were detected in the cytosolic fraction as early as at 2 days, and remained elevated throughout the experimental period. More important, though, increased levels of

cyclin D1 protein were also found in the nuclear fraction, with a maximum between 2 and 4 days, indicating that T3 has the ability to induce translocation of this cyclin from the cytoplasm to the nucleus. As expected, no evidence of sarcomeric actin in the nuclear fraction of either treated or untreated hearts was observed (Fig. 1B), confirming the purity of the subcellular fractions. The increased nuclear levels of cyclin D1 observed in T3-treated rats were associated with a decrease in the nuclear levels of the cyclin/CDK inhibitor p27 protein (Fig. 1B).

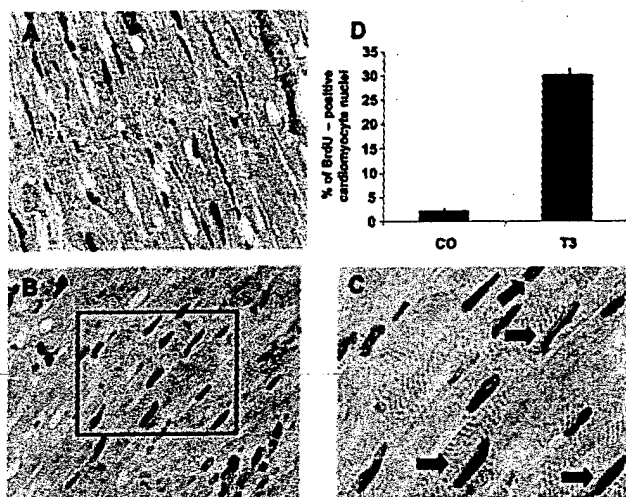
To directly establish that nuclear translocation of cyclin D1 was occurring in cardiomyocytes, immunohistochemical analysis was performed. As shown in Fig. 2, cardiomyocyte nuclei were essentially cyclin D1-negative in untreated animals, whereas T3 treatment for 4 days led to a striking increase in the number of cyclin D1-positive cardiomyocyte nuclei.

### Induction of cardiomyocyte DNA synthesis by T3

Experiments were performed to determine whether the nuclear translocation of cyclin D1 induced by T3 was accompanied by an increased replicative capacity of cardiomyocyte DNA. To efficiently score cardiomyocytes entering the S phase, a protocol was adopted in which BrdU, dissolved in drinking water, was given continuously for 7 days (17). As shown in Fig. 3A, in control rats only a few cells were BrdU-positive and most were interstitial nonmyocardial cells. On the other hand, treatment with T3 induced nuclear BrdU incorporation not only in nonmuscle cells, but also in cardiomyocytes, identified by sarcomeric actin immunohistochemical staining (Fig. 3B, C). To determine the degree of cardiomyocyte DNA synthesis, sarcomeric actin-BrdU double-stained cardiomyocytes were scored under a microscope at  $\times 1000$  magnification. As shown in Fig. 3D, a strong and highly significant increase in the number of cardiomyocyte undergoing DNA synthesis was observed after 7 days of T3 treatment (labeling index was  $30.2\% \pm 7.5$  in T3-treated rats vs.  $2.2\% \pm 2.8$  in controls). A labeling index of  $\sim 30\%$  was consistently observed in three separate experiments, demonstrating that T3 is a very strong inducer of DNA synthesis in adult cardiomyocytes. That DNA synthesis is indeed enhanced by T3 was further corroborated by findings



**Figure 2.** Immunohistochemical staining of cyclin D1 in cardiomyocyte nuclei after 4 days of T3 treatment. Cardiomyocyte nuclei positive for cyclin D1 are indicated by arrows ( $\times 200$ , counterstained with hematoxylin). CO, controls.

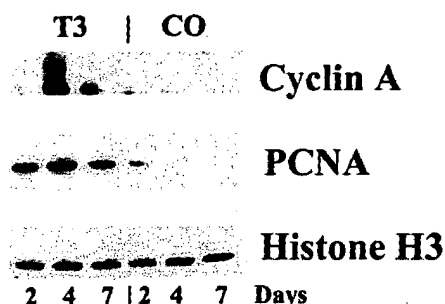


**Figure 3.** Representative microphotography of heart sections double stained for sarcomeric actin and BrdU illustrating BrdU-positive cardiomyocytes (indicated by arrows) in rats killed after 7 days of T3 treatment (B,  $\times 200$ , C, magnification  $\times 400$ ). Note distinct cross-striation of actin fibers in BrdU-positive cardiomyocytes. A) Controls. BrdU (1 mg/mL) was given in drinking water until time of sacrifice. D) Labeling index (L.I.): at least 3500 cardiomyocyte nuclei per group were scored at  $\times 1000$  magnification. L.I. was expressed as number of sarcomeric actin-BrdU-positive cardiomyocyte nuclei/100 cardiomyocyte nuclei. Results are expressed as means  $\pm$  SE of 7 rats per group. Similar results were obtained in 3 different experiments.

of increased protein levels of cyclin A, a specific marker of the S phase and of PCNA (Fig. 4), both of which showed a maximal increase after 4 days of treatment.

#### Increased cardiomyocyte DNA synthesis is not associated with heart injury

Histological analysis of hearts after 2, 4, and 7 days of treatment with T3 revealed no significant evidence of cardiomyocyte injury. Absence of tissue damage was also confirmed by the determination of serum LDH,



**Figure 4.** Western blot analysis of cyclin A and PCNA. Histone H3 was used as loading control. Total protein extracts (100  $\mu$ g/lane) were prepared from the heart and Western analysis was performed as described in Materials and Methods. Appropriate loading was confirmed by staining the gel with Coomassie blue. Each lane represents a pool of 2 samples. Similar results were obtained in 3 different experiments.

**TABLE 2.** Effect of T3 feeding for 1 wk on creatine kinase (CK), lactate dehydrogenase (LDH), and creatinine serum levels<sup>a</sup>

|         | CK (U/l)      | LDH (U/l)      | Creatinine (mg/dl) |
|---------|---------------|----------------|--------------------|
| Control | 616 $\pm$ 68  | 1775 $\pm$ 187 | 0.75 $\pm$ 0.3     |
| T3      | 290 $\pm$ 53* | 1230 $\pm$ 365 | 0.74 $\pm$ 0.2     |

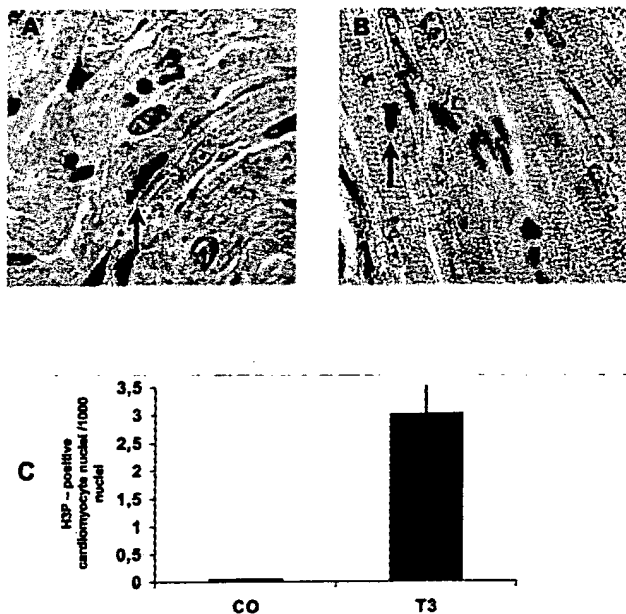
<sup>a</sup>Values are expressed as means  $\pm$  SE of 4 to 6 animals per group. \* Significantly different from controls;  $P = 0.008$ .

CK, and creatinine levels, which were either unchanged or actually lower in rats treated with T3 for 7 days (Table 2).

#### Assessment of cardiomyocyte mitoses

Histological analysis of heart sections from rats treated with T3 revealed an occasional presence of mitotic figures. We decided, therefore, to determine whether T3-induced cardiomyocyte DNA synthesis was associated with a significant increase in cell mitotic activity. As cyclin A regulates the progression through the S/G<sub>2</sub> transition of the cell cycle, and its maximal expression was found after 4 days of T3 treatment, additional experiments were performed where rats were killed after 3 or 4 days of T3 feeding. Since the duration of the M phase is markedly shorter than that of the other phases of the cell cycle, assessment of mitosis is somewhat problematic in cell populations with low levels of proliferation such as those in the heart. To ameliorate this situation, a single dose of the mitosis-blocking agent colchicine was given to the rats 3 h before sacrifice. An anti-phosphohistone-3 (H3P) antibody was also used to enhance detection of mitotic figures, since H3P on Ser<sup>10</sup> is an established marker for chromosome condensation during mitotic prophase in animal cells (20). Histological sections stained with this antibody were costained with an anti-sarcomeric actin antibody to identify cardiomyocytes. Figure 5A, B shows the presence of cardiomyocyte nuclei positive to H3P immunostaining in T3-treated rats. Several of the H3P-positive mitoses were also clearly distinguishable in sections simply stained with hematoxylin-eosin. On the opposite, almost none of the cardiomyocyte nuclei from control rats were positive to H3P. To quantitate the number of cardiomyocytes undergoing mitosis, >40,000 cardiomyocyte nuclei were scored under a microscope at  $\times 1000$  magnification, and mitotic indexes were determined (number of H3P-positive cardiomyocytes/1000 cardiomyocyte nuclei). As shown in Fig. 5C, the indexes were  $3.01 \pm 0.9$  in hyperthyroid rats killed after 3 days of treatment vs. 0.04 in controls. Similar results were obtained in hyperthyroid rats killed after 4 days of treatment (data not shown).

Results of the present study confirm that cardiac hypertrophy is an effect of T3 administration. Although T3-induced hypertrophy is usually considered a process resulting from an increase in the size of individual cardiomyocytes, our present findings provide compelling evidence that T3 treatment is also associated with



**Figure 5.** Representative microphotography of heart sections double-stained for sarcomeric actin and H3P. Rats were fed T3 or a basal diet for 3 days. A single dose of colchicine (2 mg/kg) was administered 3 h before killing. *A, B*) Cardiomyocytes undergoing mitosis are positively stained for H3P in T3-treated rats. *A*) Note distinct cross-striation of actin fibers in H3P-positive cardiomyocytes counterstained with sarcomeric actin ( $\times 400$ ). *B*) Counterstained with hematoxylin,  $\times 400$ . *C*) Mitotic index in T3-treated rats and controls (CO). At least 650 high-power fields/rat were scored. The data are expressed as number of cardiomyocyte mitoses/1000 nuclei  $\pm$  SE of 5 rats per group. Similar results were obtained in two different experiments.

activation of the cyclin D1 pathway and entry of cardiomyocytes into "S" phase and mitosis.

The question could be raised of whether the results obtained in the present study are due to a direct effect of T3 on cardiomyocytes or an indirect one mediated by stimuli deriving from some of the systemic effects of T3. Indeed, cardiac hypertrophy has been suggested to be caused in large part by increased hemodynamic load and cardiac work (21–23). This finding is best explained by the work by Klein and Hong (23), who demonstrated that when the *in situ* working heart and heterotopically transplanted nonworking heart are exposed to thyroid hormone, only the working heart (increased hemodynamic load) hypertrophies. More recently, however, it was shown that administration of  $\beta$ -adrenergic-receptor antagonist to patients with hyperthyroidism slows the heart rate without altering systolic and diastolic contractile performance, suggesting that T3 acts directly on cardiac muscle (24–26). Moreover, there are clear reasons to expect that T3 has a direct mitogenic effect: 1) T3 is able to induce cell proliferation and cyclin D1 in other organs such as liver and pancreas (16, 27), making it quite unlikely that any of the systemic effects of T3 could generate a proliferative signal or stimulus capable to affect so different organs; 2) enhanced cyclin D1 expression is associated

with cardiac enlargement and hyperplasia in adult mice, and mitotic activity during postnatal development DNA synthesis (12, 28); 3) cyclin D1 induction and shortening of the G1 phase has been shown to be induced by T3 in GC cells (29); and 4) cyclin D1 induction does not occur in other conditions of hemodynamic overload associated with cardiac hypertrophy (30). The re-entry into the cell cycle of cardiomyocytes observed after T3 treatment may therefore be a direct consequence of binding to and activation of its nuclear receptors and induction of genes involved in G1-S transition, such as cyclin D1. Studies involving the use of both agonists and antagonists of T3-receptors and  $\beta$ -adrenergic-receptor antagonists are required to clarify this point.

Whereas the present work and previous studies (16, 29) suggest that T3 induces cyclin D1 and cell proliferation, other studies have reported an opposite effect (31–35). In the work by Lin et al. (31), it was reported that, in GC cells, cyclin D1-induced repression of TR transcriptional activity is mediated by cyclin D1 recruitment of class I histone deacetylases. GC cells are T3 responsive and show increased proliferation and induction of cyclin D1 and E in response to this hormone (29). The negative regulation by cyclin D1 would appear to limit T3-transcriptional activation as a response to an active cell cycle, and would presumably occur as a secondary negative feedback to dampen induced gene expression. Lack of negative feedback, such as that observed in mice harboring a mutated thyroid hormone receptor, may explain the increased pituitary tumorigenesis (32). Moreover, more recent studies (36) have clearly shown that class I histone deacetylases promote hypertrophy in cardiomyocytes, suggesting that the cyclin D1 mediated repression of TR may not play a major role in cardiomyocytes. As to the studies reporting inhibition of cyclin D1 and proliferation of mammary epithelial cells and colon carcinoma cells (33, 34), the finding that, in different cell types (16, 27, 29), T3 induces cell proliferation that is cyclin D1-dependent suggests that the effect of T3 on cyclin D1 may be dependent on the cellular type. More important, as far as ligands of nuclear receptors are concerned, *in vitro* systems do not predict the *in vivo* response. For instance, many ligands of nuclear receptors, such as peroxisome proliferators, retinoic acid, and T3, possessing a very strong hepatomitogenic activity *in vivo*, do not induce DNA synthesis in primary culture of hepatocytes (37–39).

Manipulation of cell cycle-associated genes to promote the re-entry into the cell cycle and the proliferation of adult cardiomyocytes is a topic of considerable current interest (40). The latter is undoubtedly elicited by the possibility of eventually using the potential ability of the myocardium for regenerative growth as the basis for development of therapeutic strategies. Several studies have shown that modulation of cell cycle-associated genes/proteins can indeed induce DNA synthesis in postmitotic ventricular myocardium, *in vivo* and *in vitro* (41, 42). Constitutive expression of cyclin A or cyclins D

has been shown to lead to cardiac enlargement and hyperplasia in adult mice, and mitotic activity during postnatal development (43, 44). Moreover, heart enlargement has been shown to occur in mice overexpressing cdk2 (45) or the immediate early gene c-myc (46) and in mice knockout for the inhibitor of cyclin-CDKs complexes, p27<sup>kip1</sup> (47), suggesting that manipulation of cell cycle-related genes might help in recruiting adult cardiomyocytes into the cell cycle.

Our present results further support the latter possibility. To the best of our knowledge, they represent one of the very few demonstrations that, in an experimental model devoid of any genetic manipulation, certain agents (i.e., T3) stimulate DNA synthesis and mitosis in adult cardiomyocytes in vivo. The present results are therefore potentially significant not only in better understanding the molecular mechanisms whereby T3 induces re-entry into the cell cycle, but also for future development of therapeutic means aimed at improving cardiac regeneration. **[F]**

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